

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 0 886 141 A1

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:
23.12.1998 Bulletin 1998/52

(51) Int Cl.⁶: G01N 33/543

(21) Application number: 98810508.6

(22) Date of filing: 03.06.1998

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 23.06.1997 EP 97810399

(71) Applicants:
• C.S.E.M. CENTRE SUISSE D'ELECTRONIQUE
ET DE MICROTECHNIQUE SA
2007 Neuchâtel (CH)
• Prionics AG
8057 Zürich (CH)

(72) Inventors:
• Sigrist, Hans
CH-3309 Kernenried (CH)
• Gao, Hui
CH-2000 Neuchâtel (CH)
• Kunz, Rino
CH-8162 Steinmaur (CH)
• Duebendorfer, Juerg
CH-8032 Zurich (CH)

(74) Representative: Brulliard, Joel
c/o CSEM Centre Suisse d'Electronique et de
Microtechnique SA,
Jaquet-Droz 1
2007 Neuchâtel (CH)

(54) **Optical sensor unit and procedure for the ultrasensitive detection of chemical or biochemical analytes**

(57) This document describes an optical sensor unit and a procedure for the specific detection and identification of biomolecules at high sensitivity in real fluids and tissue homogenates. High detection limits are reached by the combination of i) label-free integrated optical detection of molecular interactions, ii) the use of specific bioconstituents for sensitive detection and iii)

planar optical transducer surfaces appropriately engineered for suppression of non-specific binding, internal referencing and calibration. Applications include the detection of prion proteins and identification of those biomolecules which non-covalently interact with surface immobilized prion proteins and are intrinsically involved in the cause of prion related disease.

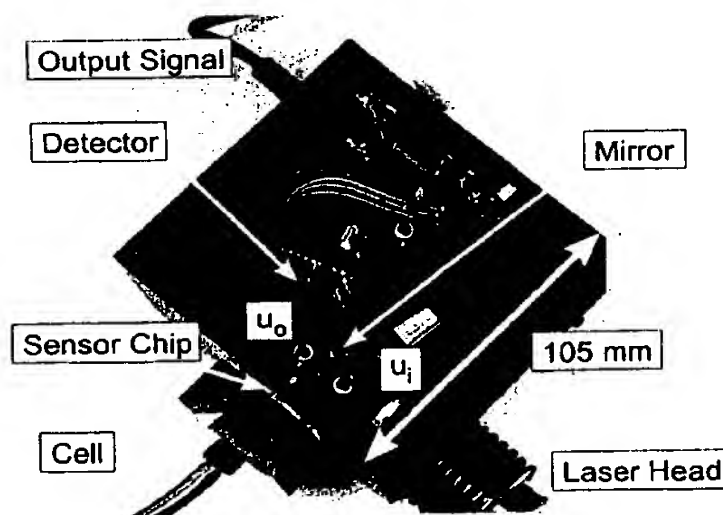


Figure 5

EP 0 886 141 A1

Description

FIELD OF THE INVENTION

The present invention relates to an optical sensor unit and to a procedure for accurate and ultrasensitive detection of chemical or biochemical analytes. An optical sensor unit is described combining several unique parts including a laser light source, an optical detection module, and an integrated optical transducer chip. The optical transducer chip consists of a disposable carrier substrate coated with a waveguiding layer onto which sensing biomolecules are bonded in pattern fashion, said sensing biomolecules being capable of interacting with chemicals or bio-molecules of an analyte solution to induce changes in the effective refractive index.

More particularly, the invention relates to the detection of prions and prion binding molecules in pico- to femto-molar concentrations with the help of antibodies, preferably monoclonal, allowing specific detection of disease-specific prion proteins on surfaces engineered for low non specific binding of bioconstituents. Conversely, the biosensor is applicable to identify ligands to prion proteins if instead of the monoclonal antibodies, recombinant or highly purified PrP is bonded to the optical chip by a one-step procedure, which can be performed even by those not skilled in the art.

More generally, the sensor and the procedure according to the invention facilitates ligand screening of chemical and biochemical libraries

BACKGROUND OF THE INVENTION

As the detection of prions and prion binding molecules is at this time of utmost importance for diagnosing and treating prion related diseases, this aspect will serve as guideline for the description of the invention. For a better understanding the abbreviations used hereinbefore and hereinafter are the following:

BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
EDTA	ethylenediaminetetraacetic acid
GPI-anchor	glycolipid-anchor which "ties" PrP to the outer monolayer of the cell membrane
HEPES	hydroxyethyl-piperazineethane sulfonic acid
IO	integrated optical
PBS	phosphate-buffered saline
prion	proteinaceous infectious particle; the infectious agent of prion diseases, supposedly consisting at least of PrP ^{Sc} and maybe another (other) yet unknown molecule(s)
PrP	prion protein; refers to the common amino acid sequence rather than to a distinct conformation of two prion protein isoforms

PrP^C

ms

a normal host prion protein of unknown function; apparent molecular weight 33-35 kDa, same amino acid chain, and same glycosylation at two asparagine residues as PrP^{Sc}. This prion protein is after proteinase K treatment fully digested.

PrP^{Sc}

the disease-specific, abnormal isoform of PrP^C, with the same amino acid chain, apparent molecular weight 33-35 kDa, glycosylated at two asparagine residues, is after proteinase K treatment shortened to a 27-30 kDa C-terminal fragment. Species-specific PrP^{Sc} isoforms may term: human PrP^{Sc} (instead of PrP^{CJD}), bovine PrP^{Sc} (instead of PrP^{BSE}) etc.

rb PrP

recombinant bovine prion protein (amino acids 25 to 242 of the bovine PrP gene) expressed in E. coli comprising the bovine PrP open reading frame except for the N-terminal signal sequence and the C-terminal GPI-anchor sequence; both are cleaved off during cellular processing. Since this protein is not glycosylated it has a molecular weight of 23 kD.

SPOT

smart planar optical transducer

The references to the prior art reported hereinafter will be termed by the name of the first author and the publication year as mentioned below:

- Basler, K., Oesch, B., Scott, M., Westaway, D., Walchli, M., Groth, D.F., Mc Kinley, M. P., Prusiner, S.B., and Weissmann, C. (1986). Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell* 46, 417-428.
- Borchelt, D.R., Scott, M., Taraboulos, A., Stahl, N., and Prusiner, S.B. (1990). Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. *J. Cell Biol.* 110, 743-752.
- Bueler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.P., DeArmond, S.J., Prusiner, S.B., Aguet, M., and Weissmann, C. (1992). Normal development and behaviour of mice lacking the neuronal cell surface PrP protein. *Nature* 356, 577-582.
- Dübendorfer, J., Kunz, R.E., Mader, E., Duveneck, G.L. and Ehrat, M. (1996) "Reference and Sensing Pads for Integrated optical Immunosensors," *Proc. SPIE*, Vol. 2928, 90 - 97.
- Gao, H., Kisling, E., Oranth, N., and Sigrist, H. (1994). Photolinker-polymer-mediated immobilization of monoclonal antibodies, F(ab')₂ and F(ab') fragments. *Biotechnol. Appl. Biochem.* 20,

251-263.

Gao, H., Sanger, M., Luginbuhl, R., Sigrist, H., Immunosensing with photoimmobilized immunoreagents on planar optical wave guides. *Biosensors & Bioelectronics* 10 (1995) 317-328.

Heding, A., Gill, R., Ogawa, Y., De Meyts, P., Shymko, R.M. (1996). Biosensor measurement of the binding of insulin-like growth factors I and II and their analogues to the insulin-like growth factorbinding protein-3. *J. Biol. Chem.* 271, 13948-52.

Kitamoto, T., Mohri, S., and Tateishi, J. (1989). Organ distribution of proteinase-resistant prion protein in humans and mice with Creutzfeldt-Jakob disease. *J. Gen. Virol.* 70, 3371-3379.

Korth, C., Stierli, B., Moser, M., Streit, P., Oesch, B. (1997) Immunological detection of prions. European Patent application No. 97102837.8.

Kunz R.E., "Process and device for determining quantities to be measured by means of an integrated optical sensor module", U.S. Patent No 5,442,169.

Kunz R.E., "Integrated Optical Sensors based on Hard Dielectric Films on Replicated Plastic Substrates", Proc. 8th Eur. Conf. Integrated optics ECIO,97, Stockholm, Sweden, April 2-4, 1997, pp. 86-93.

Kunz, R. E. (1996). "Integrated Optical Modules for Chemical and Biochemical Sensing". Bulletin of the Swiss Microtechniques Association (ASMT) 20, 48-53.

Kunz, R.E. and Dubendorfer, J. (1995). "Miniature Integrated Optical Wavelength Analyzer Chip". *Optics Letters* 20, 2300-2303.

Kunz, R.E., and Dubendorfer, J. (1996). "Novel Miniature Integrated Optical Goniometers". Proc. EUROSENSORS X, Conference on Solid-State Transducers 3, 905 - 908.

Kunz, R.E., Duveneck, G., and Ehrat, M., (1994). "Sensing Pads for Hybrid and Monolithic Integrated Optical Immunosensors", Proc. SPIE 2331, 2-17.

Kunz, R.E., Edlinger, J., Sixt, P., and Gale, M.T. (1995) Replicated Chirped Waveguide Gratings for Optical Sensing Applications. *Sensors and Actuators* 47, 482-486.

Kurschner, C. and Morgan, J.I. (1995). "The cellular prion protein (PrP) selectively binds to bcl-2 in the

yeast two-hybrid system. *Molecular. Brain Research.* 30, 165-168.

Lukosz, W. and Tiefenthaler, K., "Directional switching in planar waveguides effected by absorption-desorption processes." IEE Conf. Publ. 227, 152-155 (1983).

Oesch, B. (1994). "Characterization of PrP binding proteins". *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 343, 443-445.

Oesch, B., Westaway, D., Walchli, M., McKinley, M., P., Kent, S.B., Aebersold, R., Barry, R.A., Tempst, P., Teplow, D.B., and Hood, L.E. (1985). "A cellular gene encodes scrapie PrP 27-30 protein". *Cell* 40, 735-746.

Oesch, B., Teplow, D.B., Stahl, N., Serban, D., Hood, L.E., and Prusiner, S.B. (1990). "Identification of cellular proteins binding to the scrapie prion protein". *Biochemistry* 29, 5848-5855.

Serban, D., Taraboulos, A., DeArmond, S.J., and Prusiner, S.B. (1990). "Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins". *Neurology* 40, 110-117.

Stahl, N., Baldwin, M.A., Teplow, D.B., Hood, L., Gibson, B.W., Burlingame, A.L., and Prusiner, S.B. (1993). "Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing". *Biochemistry* 32, 1991-2002.

The disease-specific forms of the prion protein (PrP^{Sc}, PrP^{BSE}) are part of the infectious particle causing transmissible neurodegenerative diseases like scrapie in sheep, bovine spongiform encephalopathy in cattle or Creutzfeldt-Jakob disease in humans. PrP^{Sc} as well as PrP^{BSE} differ from the normal cellular prion protein (PrP^C) by their relative protease resistance (Oesch, 1985). The molecular changes leading to this difference in physicochemical properties are unknown. Protease-resistant PrP^{Sc} and infectivity to date have not been separated, leading to the proposal that the infectious particle would be composed of specifically altered PrP molecules. The primary amino acid sequence of PrP^{Sc} is identical to that predicted from its cDNA sequence or genomic nucleic acid sequence (Stahl, 1993) and the infectious particle does not encode an altered PrP gene (Oesch, 1985). In cell culture, PrP^C is converted into PrP^{Sc} posttranslationally (Borchelt, 1990). However, no differences in covalent modifications of PrP^{Sc} and PrP^C were observed by mass spectrometry (Stahl, 1993). The lack of a molecular explanation for the observed differences between PrP^{Sc} and PrP^C led to the proposal that the PrP isoforms differ in their conformation (Basler, 1986). The relative protease resistance of PrP^{Sc}, e.g.

PrP^c being fragmented by proteinase K and PrP^{Sc} being partially resistant to the action of proteinase K, is at this time the usual way to distinguish the two forms of PrP (Serban, 1990).

PrP^{Sc} is currently detected by Western or dot blotting. After protease digestion, the molecular weight of PrP^{Sc} is reduced from 33-35 kDa to 27-30 kDa (PrP 27-30). This characteristic change in molecular weight is detected by Western blotting and serves as a hallmark of PrP^{Sc}. As an alternative, PrP 27-30 can be detected on dot blots (Oesch, 1994; Korth, 1997). Native PrP 27-30 is not recognized by antibodies, whereas the denatured form of the protein interacts with antibodies. (Serban, 1990). Circumstantial evidence indicates that the epitopes in PrP^c and PrP^{Sc} differ in accessibility. Previously, it has been attempted without success to generate antibodies which specifically recognize PrP^{Sc}, thus limiting the immunological detection methods to procedures including denaturation of samples prior to detection. However, (Korth, 1997) described monoclonal antibodies (see below) that do allow detection of native PrP^{Sc} and, specifically, PrP^{BSE}.

A receptor for PrP transducing biological effects of PrP has not been found yet. Until now, various proteins have been shown to interact with PrP: glial fibrillary acidic protein (GFAP); (Oesch, 1990), bcl-2 (Kurschner, 1995). All of these proteins are intracellular proteins which contrasts with the cell surface location of PrP. Even though there may be a role for these PrP binding proteins, in particular bcl-2, in the neurotoxic effects of PrP^{Sc} it is postulated that a cell surface protein should exist which binds to PrP. Despite the scientific investigations summarized above, there is still a need for a detection system which impairs as little as possible the conformation of the molecules or biomolecules to be detected.

Indeed, known analyte detection techniques have their limitations in terms of sensitivity and quantification, the procedure involving several steps, the necessity of detection of labelled components and the overall duration of the test procedure.

With the present invention, these shortcomings are overcome by the use of biosensors which allow the registration of molecular interactions at the surface of integrated optical chips. The basic principle of these biosensors is to measure the change in the effective refractive index for the guided waves on an optical chip when a ligand interacts non-covalently with molecules bonded to the surface of the respective chip. The specificity of the interaction is ascertained by the use of molecules such as antibodies immobilized on the surface of integrated optical chips. The described PRP^{BSE} biosensor detection system is based on an optical transducer in combination with miniature integrated optical sensor devices. Optical waveguide sensing provides the features of exceptional high sensitivity and label free detection of analytes within the evanescent field of incoupled laser light. Requirements for high selectivity, reduction of non-

specific binding and multiple sensing on a single waveguide surface are accomplished by appropriate sensor surface bioengineering combined with multidomain photobonding. The use of multidomain analysis on integrated optical chips dramatically simplifies the instrumental features of the detection system. Multidomain analysis on a single chip enables integrated calibration and referencing. The described prior detection system in combination with unique monoclonal antibodies is used to detect PRP^{BSE} with high sensitivity and high speed. It allows to screen large numbers of samples.

With the same detection system, on the other hand, biomolecules interacting with PrP can be identified. For this purpose, instead of covalently bonding monoclonal antibodies to PrP, rb PRP or highly purified native PrP, PrP^c or PrP^{Sc} is immobilized on the surface of the optical chip. Since applied detection principles do not require labelling of a probe, it is exquisitely suited to search for (an) elusive "PrP receptor(s)" which is (are) currently unknown but postulated to be essentially involved in the modulation of PrP function as well as in the infection of cells by prions.

The described biosensor system further suits the screening of chemical libraries in search for potential ligands which interact with molecules bonded to optical chips. For example, PrP bonded on an optical chip allows to screen for chemical ligands capable of preventing neurotoxic and infectious properties of prions.

OBJECT OF THE INVENTION

It is the object of the present invention to overcome the drawbacks and failures of prior art like complex multistep procedures, limited sensitivity, necessity of label-based analyte detection and necessity of sophisticated equipment. These limitations are addressed in the present invention, which provides an accurate and ultrasensitive detection system for biological ligands, especially prion proteins and proteins which interact with prion proteins, to be used in the diagnosis of prion diseases, or any other biological state involving detection of molecules in biological fluids or tissue homogenates and requiring high sensitivity.

SUMMARY OF THE INVENTION

The present invention describes a novel sensor system which is able to detect and quantify pico- to femtomolar concentrations of chemicals or bio-molecules in analytes of biological origin in real fluids. Analytical limitations of hitherto systems are overcome by combining the exceptional sensitivity of a new design for simplified integrated optical detection on disposable sensor chips with the intrinsic specificity of sensing bioconstituents and photobonding and biopattern technologies enabling both array detection and integrated calibration. Teachings include the design of the biosensor system and its

essential components: the design and fabrication of multi-array biosensor chips and the preparation and characteristics of high-affinity disease-specific bioconstituents. General use of the biosensor system for on-site application is exemplified with the detection of PrP^{BSE} in homogenates of brain and the detection of molecular components participating in prion related diseases, e.g. identification of PrP^{BSE} binding biomolecules.

The present invention concerns the layout and description of the modular system components of the analytical instrument. In its final form, the instrument is portable and at least consists of a laser light source, an optical detection module and an integrated optical transducer chip. This instrument can be extended with an effector system, such as a fluid handling system for allowing the selection of individual samples, solvents or washing solutions, and with an appropriate computer-like component for data storage, processing and presentation.

Sensor pad illumination is achieved by directing the radiation from sources such as laser diodes in the red or near infra-red wavelength region (e.g. Sharp LT026MDO, $\lambda \approx 785$ nm), either directly or via optical fibers. The optical output signal is detected by one- or two-dimensional detector devices, such as charge coupled devices (CCDs), photodiode arrays (PDAs), such as Hamamatsu S5463, or position sensitive devices (PSDs). The signal is evaluated and stored in digitized form and prepared for transmission to the computer component, e.g. RS232 interface.

In a preferred embodiment, the optical transducer chip consists of a high refractive index dielectric waveguiding film (e.g. TiO₂, Si₃N₄, mixtures of TiO₂ and Si₃N₄, Ta₂O₅, ZrO₂, HfO₂, Nb₂O₅), coated on a previously structured substrate (e.g. polycarbonate or polystyrol). The substrate is structured with (chirped) grating pads by means of procedures, as e.g. hot-embossing or injection moulding. The grating pads fulfill the tasks of coupling the light into the waveguiding film, of on-chip signal generation and of coupling the light out of the waveguide and directing it to the detector. A typical size of one sensor pad is in the order of a few mm². Arrays of sensor pads can be accomplished by placing several pads next to each other. Using sensor pad arrays is advantageous for chemical multicomponent analysis and on-chip referencing. Preferred shapes of the biosensor chip are rectangular (card) or circular (disc). If the transducer is in the form of a disc, the disc is rotated on a central axis and contains optical on-chip elements for speed control and pad-specific information (e.g. position, sensing layer etc.). In the disc format, the sensing pads are arranged in concentric circles or in spiral arrangements. Either each pad is addressed consecutively, or several, or all pads can be interrogated in parallel by means of single or multiple beam illumination, e.g. by means of light source and detector arrays.

The sample holder consists either of an open cell allowing manual sample application, or it consists of a

closed cell with tubings (e.g. Teflon, Peek) and a suitable liquid handling system.

The present invention concerns further a process for chip surface engineering including bonding of biomolecules, such as PrP, monoclonal or polyclonal antibodies, mixtures of monoclonal antibodies or other proteins which recognize specific epitopes on the target proteins (e.g. PrP). In a preferred embodiment, these proteins or antibodies are covalently immobilized on the waveguiding surfaces by means of photolinker polymer mediated photobonding or by oriented immobilization of analyte recognizing receptor molecules, genetically engineered receptor molecules or fragments of analyte recognizing immunoreagents through surface grafting with e.g. bifunctional photoactivatable maleimides. As an alternative, antibodies or other proteins are immobilized on the chip surface by absorption.

The present invention concerns further the process of engineering of the optical transducer surface with sensing biomolecules appropriate for analyte detection. Using mask-free or mask-assisted patterning procedures, the individual pads on the optical transducer surface are individually functionalized with selected molecules and varying compositions of biomolecules. For referencing, given sets of pads are modified with non-specific interacting analogs of the specific analyte-binding molecules, and on chip referencing is attained by sets of pads which provide increasing amounts of specific analyte binding molecules. Differential analyte binding kinetic data recorded from these calibration pads allow on-chip analyte calibration with reference to established binding constants.

The present invention concerns further a process, in which non-specific binding of bioconstituents present in the analyte fluid is suppressed by surface coating with polyethylene glycol, antibodies which do not interact with the ligand, polycarbohydrates, carbohydrates presenting low molecular weight photolinkers, or mixtures of the components mentioned.

The invention concerns further a procedure for verifying the proper functioning of the integrated optical transducer in situ, before and after each measurement by varying the incidence and/or the wavelength of the light.

The present invention concerns further a process in which the output signal light of a first optical sensor unit can be used to regulate, optionally by means of a computer, a second effector system and thus allowing the automation of biochemical reactions involving many single sequential or parallel steps.

The present invention concerns further a combination of the analytical detection system, the optical biosensor module bonded with monoclonal antibodies to PrP able to detect PrP in a fluid guided to the optical sensor unit.

The present invention concerns further a combination of the analytical detection system, the optical biosensor module with photobonded or oriented immobi-

lized PrP able to detect biomolecules which non-covalently interact with said immobilized PrP.

The present invention concerns further a process for the identification and medical use of those biomolecules which can be detected and identified after non-covalent binding to surface-bonded PrP if said biomolecules inhibit the propagation of PrP destabilization or reverse PrP unfolding.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1 is a schematic representation of two IO sensor system types: (a) conventional arrangement (type I) and (b) novel compact miniature detection unit (type II);
- Figure 2 shows in (a) the photolinker polymer mediated immobilization of biomolecules, where the photolinker consists of modified bovine serum albumin (TBSA), and in (b), a preferred immobilization procedure suppressing non-specific bonding;
- Figure 3 is a diagram showing the successful binding of antibodies 6H4 to immobilized PrP. Recombinant bovine PrP was photoimmobilized on sensor chips. Incubation with PBS shows the baseline. After the addition of antibody 6H4 a change in adlayer thickness (Δh_1) is observed indicating binding of the antibody. A wash with PBS does not reduce the observed response;
- Figure 4 shows in (a-c) a miniature sensor chip based on a dual pad chirped grating pad structure, in (d) a corresponding single chip sensor array, and in (e) a SPOT in a disc format;
- Figure 5 shows a compact sensor module prototype based on a replicated SPOT chip.

DETAILED DESCRIPTION OF THE INVENTION

In the following detailed description the spirit and scope of the invention will become more clearly explained.

The monoclonal antibodies

A monoclonal antibody according to the invention is defined as a biomolecule which binds to epitopes of prion proteins, whether they are in soluble or insoluble form in various tissue specimens, such as homogenates or sections of brain, spleen, tonsils, white blood cells, or others, and body fluids, such as blood, cerebrospinal fluid, saliva, urine, or others. The present mABs bind to epitopes of amino acids in a row or to epitopes of amino acids on different loops of the three-dimensional structure of native PrPs, which are spatially close to each other. A particular group of the present antibodies binds on-

ly to native disease-specific PrP and not to native normal PrP. Particular antibodies, termed 6H4, 34C9, 15B3 according to the invention, are more detailed in (Korth, 1997).

The term monoclonal antibody comprises also chimeric monoclonal antibodies having similar properties, which are derived from different animals, such as human/mouse chimeric antibodies or any other chimeric molecule comprising the antigen-binding part of the monoclonal antibody (idiotype) with other molecules such as antibody fragments of other monoclonal antibodies or enzymes.

A fragment of a monoclonal antibody comprising the binding part of the monoclonal antibody (idiotype) is likewise capable of specifically binding the antigen and is termed F(ab) or F(ab')₂, depending on whether the monoclonal antibody is digested with papain or pepsin, respectively.

A synthetic antibody or fragments thereof is designed according to the amino acids or substituted homologous amino acids composing the idiotype responsible for binding the antigen. Homologous amino acids are defined as exchanges within the following five groups: 1. Small aliphatic, nonpolar or slightly polar residues: alanine, serine, threonine, glycine, proline; 2. Polar, negatively charged residues and their amides: aspartic acid, asparagine, glutamic acid, glutamine; 3. Polar, positively charged residues: histidine, arginine, lysine; 4. Large aliphatic, nonpolar residues: methionine, leucine, isoleucine, valine, cysteine; 5. Large aromatic residues: phenylalanine, tyrosine, tryptophan.

The antibodies and fragments thereof are essential tools for immunological detection procedures based on the binding of the prion protein to the presented monoclonal antibodies in an antigen-antibody complex. The monoclonal antibodies of the invention react with recombinant bovine PrP as well as native or denatured PrP^c and PrP^{Sc}, whether they are in soluble or insoluble state. The monoclonal antibodies react furtheron with PrP from different species, for example humans, hamsters, pigs, sheep, cattle and mice.

Anti-idiotypic antibodies

The invention concerns further anti-idiotypic antibodies which are antibodies that bind with the binding region (idiotype) to the binding region of the original monoclonal antibody. The anti-idiotypic antibody mimicks features of the original antigen, in this case features of PrP. Anti-idiotypic antibodies are raised as polyclonal antibodies (serum) or monoclonal antibodies from animals immunized with the preferred antibodies according to the invention. Anti-idiotypic antibodies are valuable tools in detecting and blocking interactions of the original antigen (PrP), particularly interactions with receptors, and can therefore be used in prevention and therapy of prion diseases.

The recombinant bovine prion protein

The present fragment of the prion protein PrP^c was purified to a homogeneity of > 98% and is described in detail in (Korth, 1997). It can exist either in oxidized or reduced form. In the oxidized form, there is a single -S-S-bridge whereas, in the reduced form, two SH groups are present instead. The oxidized form has a molecular weight of 23676.8 kD, and the reduced form 236886.1 kD, as determined by mass spectroscopy.

A native prion protein PrP is the prion protein in a fully folded state, i.e. the three-dimensional structure is present. Only in the native, i.e. folded state, PrP isoforms are different (normal native vs. disease-specific native PrP).

A denatured prion protein is the prion protein in the unfolded state. This is usually achieved by the addition of chaotropic substances, such as urea or guanidinium hydrochloride. In the denatured state, both PrP isoforms are irreversibly the same, even if they have been normal native or disease-specific native before.

An antigen-antibody complex is a physical attachment of an antibody, or fragment thereof, with the corresponding antigen by intermolecular forces because the surfaces match in a unique way. The matching surface on the antibody is called idiotype and the surface on the antigen is called epitope.

The biosensor chip and module

A miniature biosensor chip provides a versatile platform for biomolecule binding, quantitative detection of biomolecules and for selective retention of biomolecules. The disposable biosensor transducer chip (figure 4) consists of a high refractive index dielectric waveguiding film (F) deposited on a previously structured substrate (S). The substrate is structured with grating pads (G1, G2) by microstructuring technologies including hot embossing or injection moulding. The grating effects appropriate incoupling of light, signal generation and out-coupling of the light. The size of the sensor pads may vary from tens of mm² to submillimeter dimensions. The pads are arranged pairwise or serial, allowing individual and group referencing, respectively. The array sensor platform is essential for multicomponent analysis and on-chip referencing. Preferred shapes of the biosensor chip are rectangular (figure 4.d) or circular disc-shaped (figure 4.e) forms. Multi-arrays on rectangular platforms can be addressed by fibre guided light, originating from a single source or from multiple light sources. Disc-shaped optical platforms use CD technologies to address individual pads, enabling high repetitive reading frequencies and thus, fast kinetic measurements.

Essential modular components of the instrument include the chip holder (figure 5), a fluid handling system which provides controlled analyte and buffer throughput. The fluid handling system is designed for high analyte surface interaction and minimal system surface ex-

posure. Guided light from laser light source (Laser Head) impinges on the grating structures (Sensor Chip) and generated output signals are recorded by photodiode arrays (Detector).

Bonding of biomolecules to the optical transducer surface

Biomolecules are known to interact with material surfaces in accordance with the physico-chemical properties of the material and the biomolecule surface. Appropriate surface engineering is introduced in order to control the physical processes, enhance uniform biomolecule surface interactions and promote, optionally, covalent biomolecule binding. Covalent biomolecule binding is advantageous since it provides the means for extensive washing and thus reduction of non-specific binding of system constituents. Non-specific binding can be suppressed or prevented by introducing agents on the transducer surface which repel biomolecules. Polyethylene glycol (PEG), carbohydrates, the polar head groups of lipid bilayers, as well as selected protein layers may serve to suppress non-specific binding. Preferred processes for biomolecule binding are those which are technically easy to perform, lead to covalent binding and can be utilized for multicomponent binding. Photobonding is one of the processes which per se, or in combination with microdispensing methods, allows addressable printing of biomolecules on individual sensing pads. Photobonding technologies enable experimentally easy formation of pad to pad gradients providing different biomolecule densities at individual sensor pad surfaces. A preferred method of photobonding is the photopolymer mediated immobilization of biomolecules with light. The process includes mixing of the biomolecule(s) to be immobilized with a photoactivatable polymer prior to the deposition on the transducer surface. Upon exposure to light, covalent bonds are formed between the photopolymer, the biomolecules and the surface effecting photoimmobilization. A comparable process leads to the covalent bonding of a low molecular weight crosslinker to the transducer surface. If photoactivatable hetero-bifunctional crosslinkers are immobilized on the surface, the prerequisites are established for oriented macromolecule binding. For example, photobonding of an aryldiazirinomaleimide crosslinker provides a grafted surface to which biomolecules with reactive thiol function covalently attach. As an alternative to covalent binding of biomolecules to the chip surface, they are passively absorbed to the chip surface. Preferred conditions are concentrations of 0.5 µg / mm².

Detection procedure of prion disease with biosensors

An immunological detection procedure for prion disease, especially BSE in cattle and scrapie in sheep, whereby disease-specific PrP^{Sc} protein in biological

material of an animal or human comprises treatment of a first probe of said material with a monoclonal antibody to PrP and detecting the mixed PrP^c/PrP^{Sc}-antibody complex, treating a second probe of said material first with proteinase K and then with the monoclonal antibody, detecting the PrP^{Sc}-antibody complex and analysing the results of both probes. A monoclonal antibody termed 15B3 is used to detect PrP^{Sc} in a PrP^{Sc}-antibody complex without prior protease-digestion of the tissue specimen to be examined. Biological material can be insoluble or soluble, and of any part of the body, e. g. from the brain in which case it is used in form of a homogenate or tissue sections, or any body fluid, e. g. cerebrospinal fluid, urine, saliva or blood. In the case of body fluids, fluid-resident cells, e.g. white blood cells in the case of blood expressing PrP, can be purified and analyzed.

The detection of the PrP^{Sc}-antibody complex is carried out on a smart planar optical element. This smart planar optical element is covalently coated with the described monoclonal antibodies. Analyte solutions are then guided to the sensing area of the chip via an appropriate fluid system. Analyte binding of the surface is recorded by registration of effective refractive index changes.

The present immunological detection procedures allow the diagnosis of prion diseases. With the tools of the present invention, tissue sections, tissue homogenates or body fluids of prion-infected animals, such as BSE-diseased cattle or humans having the CJD, can be screened for the presence of the protease-resistant, disease-specific isoform of the prion protein in its native form, be it soluble or insoluble.

Instead of directly analysing the binding of prions to antibodies immobilized on the chip surface, a competition assay is described. A protein ligand for anti-PrP antibodies is immobilized on the chip surface. Tissue homogenates or body fluids are incubated with anti-PrP antibodies. The mixture is then reacted with the molecules on the chip surface. Residual free antibodies will then bind and elicit a signal. In the case of an animal or human with prion disease, part or all of the antibody will already be bound to PrP in the sample, thereby preventing the binding of the antibody to the chip surface, effectively reducing the signal observed with a sample from a normal animal or human. Reduction of the signal is therefore indicative of prion disease.

Method for the identification of ligands, especially receptors with miniature biosensor chips

Recombinant PrP is bonded on the optical chip. Homogenates containing presumed ligands are guided through the fluid system onto the optical chip. Molecules binding to the immobilized recombinant PrP increase the effective refractive index. Non-specifically binding bioconstituents are first removed by washing with appropriate solvents. Specifically binding ligands are then

removed from the surface by selected desorption procedures. Ligands present in the eluate are identified and characterized.

Method for screening chemical or pharmaceutical libraries for the identification of small compound molecules useful for therapy

Recombinant PrP or highly purified PrP is bonded on the optical chip. Homogenates containing presumed ligands are guided through the fluid system onto the optical chip. Molecules binding to the immobilized recombinant PrP alter the effective refractive index. The change of the effective refractive index indicates molecule binding.

Method for an integrated chip able to detect several ligands in parallel

Smart planar optical elements are produced as described. Ligands or particularly monoclonal antibodies recognizing given molecules are bonded as described on these chips on distinct pads. The analyte to be examined is guided onto the chip. A laser or several laser beams are directed to the pads and the system detects the change of the effective refractive index at these pads. This allows performance of highly sensitive quantitation of various molecule concentrations in a complex analyte solution.

Small portable analytical instrument

The described compact biosensor unit is designed as a portable autonomic unit capable of detecting one type or several types of molecules in analyte solutions or body fluids. Arrays of individual sensing areas are bio-engineered with different amounts of one type of biomolecules (intrinsic gradients) or the surfaces contain varying amounts of several components. The portable analytical instrument can be used for outdoor or on-site testing.

The following examples serve to illustrate particular embodiments of the invention but they should not be considered a limitation thereof. The immunological procedures are outlined for the diagnosis of BSE in cattle, however, these procedures can also be applied for prion diseases in humans or animals, such as sheep, hamsters or mice. Furthermore, the described procedure can be applied for other diseases of human and animals utilizing appropriate sensing biomolecules and ligands.

EXAMPLES

Example 1: Design, fabrication and testing of biosensor chips

Detection of immunological interactions between recombinant bovine PrP and monoclonal anti-PrP anti-

bodies is accomplished by combining optical waveguide detection and light-addressable photobonding. In view of the exceedingly small quantities of analytes in test samples (10^5 to 10^8 molecules), biosensor technologies must meet the requirements of high sensitivity and low non-specific binding. Designed for routine analysis, the detection system preferably consists of few components, fast in response (measuring time in the order of minutes or shorter) and suitable for mass production. The availability of disposable sensor devices, combined with multiple sensing and referencing pads on a single device is advantageous for diagnostic analysis.

I. Optical waveguide design and fabrication

To adapt the resolution and dynamic range of the sensor chip to the needs of diagnostic application, corresponding substrates are fabricated. The grating structures are written by an e-beam pattern generator on quartz plates covered with chromium and a thin resist layer. After development, the pattern is transferred into the chromium and then by etching into the underlying quartz substrate to produce a surface relief grating with a depth of 5 to 10 nm. The pattern relief is electroformed to a nickel shim from which replicas are fabricated by hot embossing into a 250 μm thick polycarbonate foil (Röhm Europlex PC 99510). These embossings are generally 50x50 mm² in size and contain several grating regions. The waveguide is then fabricated by depositing thin TiO₂ films (typically about 160 nm thick) on the pre-structured substrates by a modified d.c.-magnetron sputtering process. After cutting, the chips are glued on-to glass substrates for stability reasons.

New nonlinear chirps of the grating lines are also fabricated allowing (at low resolution) highly sensitive signal analysis. A first layout is designed for a dual pad sensor with one sensing and one reference pad. For the analysis of complex systems, more than two pads are preferable (multichannel and multicomponent analysis). Standard industrial processes, as e.g. compact disc (CD) injection moulding, are preferred chip fabrication modes.

II. Optimization of antibody photobonding in view of effective reduction of non-specific binding in real fluids (e.g. brain homogenates)

The experiment described in Figure 3 was carried out by photobonding of PrP or anti-PrP antibodies to the chip surface by a method similar to the one described by (Gao, 1995). Alternatively, in order to maximize the density of antibody on the surface, maleimido-anyldiazine mediated antibody immobilization procedures were applied. To achieve this, F(ab')₂ fragments are prepared. The disulphide bond in the constant region is selectively reduced and the free SH groups are used to immobilize the F(ab') fragments in a directed manner, i.e. the binding site pointing towards the liquid phase. This

procedure ensures maximal packing of binding domains on the chip surface.

III. Optimization of assay parameters for the detection of PrP in brain homogenates

The antibody 6H4 recognizes also mouse PrP. This particular feature allows to control for the specificity of the system by using mouse brain homogenates from either wild type or PRP^{0/0}. Brain homogenates from either type are prepared under different conditions. The following buffers are used: 0.25 M sucrose, 20 mM HEPES, pH 7.0 or Sucrose/HEPES buffer containing in addition 2% N-lauryl sarcosine, 15 mM EDTA, 3 mM dithiothreitol. Sucrose/HEPES/EDTA buffer in combination with the following detergents were tested: Triton X-100, octyl glucoside and Tween 20 at various concentrations. In addition, DNase was added into the homogenization buffer in order to reduce the viscosity due to DNA. These experiments showed the best calibration conditions that have to be used for an optimal signal to noise ratio. Along the same line recombinant PrP was reconstituted into PRP^{0/0} mouse brain homogenates which allowed to directly compare the signal of a specific amount of PrP in its pure form and mixed into homogenates. Since proteinase K digested brain homogenates were used for the detection of PrP^{BSE} recombinant PrP was also reconstituted into proteinase K-digested bovine brain homogenates which allowed to optimize the detection system for the actual conditions used for the detection of PrP^{BSE}.

Example 2 : Building of a biosensor module

Since PrP^{BSE} only occurs in combination with infectious particles, it is important to have a mobile biosensor module which can be used under biosafety conditions P2. A new compact and miniaturized IO sensor module was constructed and designed. A schematic drawing of such a module is shown in Figure 5.

The complete module consists of three distinct sub-modules. The tasks of the submodules are *illumination* (i.e. to provide the optical input), *signal transduction* into an on-chip measuring variable and *signal evaluation*. In the sensor module shown in Figure 5, the electrical input connector (laser driver) powers a laser diode (CD laser diode at 785 nm) for illumination of the sensor chip. A fluid handling system (tubings in the back) is used for analyte application. The sensor output signal is detected using an one-dimensional photodiode array. In this example, the electrical output signal is fed into a laptop computer (RS232 interface) via the connector to the right of the module.

Example 3 : Detection of PrP^{BSE}

The functional biosensor module as described above was used to test the detection of bovine PrP^{Sc}.

Specifically, the chips carrying either immobilized 6H4 or 15B3 were incubated with proteinase K-digested brain homogenates from normal or BSE-sick cattle. From Western blotting experiments, it is known that about 10-30 % of total PrP in a BSE-infected brain correspond to PrP^{BSE}. Using the above described procedures, antibodies immobilized on the chips were incubated with proteinase K digested brain homogenates. No signal was observed in normal brain while specific binding was detected in homogenates from BSE-sick animals. For antibody 15B3, undigested homogenates were used and a signal was detected only in homogenates from BSE-sick cattle.

This method was further tested for the detection of PrP^{BSE} in organs other than brain. The exquisite sensitivity of the biosensor system allowed to detect PrP^{BSE} in spinal fluid, lymphoid organs, blood, saliva or urine. Since the antibody 6H4 also recognizes mouse and sheep PrP, it was also used to test tissues from these animals. For sheep, it is known that placenta contains infectious material allowing to analyze the presence of PrP^{Sc} in order to correlate infectivity and occurrence of PrP^{Sc}. In the mouse system, various tissues, such as spleen or intestine, are known to contain PrP^{Sc} (Kitamoto, 1989). These experiments allowed to detect the amount of PrP^{BSE} in various tissues and therefore allow also to estimate indirectly whether they may contain infectious substances.

The following description details the preferred procedure to achieve suppression of non-specific binding (figure 2.b). The signal generated by specific interaction of the ligand binding molecule (e.g. anti-prion antibody) with the analyte PrP in real fluids can be as small as 8 to 20% of the signal produced by constituents of the real fluid. These latter constituents interact non-specifically with the engineered or pristine surface and thus, generates a sensor response. Suppression of such non-specific binding is achieved by including molecular components in the mixture used for surface engineering. Molecules added to the mixture used for surface bioengineering and photobonding include polyethylene glycol (PEG), amino-PEG, carboxy-PEG, polycarbohydrates (dextran, inulin), phospho-rylcholine containing polymers, selected components of the real fluid and mixtures thereof, fractions of complete control serum or homogenate not containing the analyte. In general terms, any molecular species effective in the suppression of non-specific binding is mixed with the analyte-binding molecule and the photolinker polymer. Then the mixture is spread on the transducer surface and photobonded. For the detection of recombinant PrP in a non real fluid, the preferred components used for surface engineering include T-BSA (65% w/w), PEG (10% w/w) and monoclonal anti-PrP (25% w/w). For the detection of PrP in mouse brain homogenate, the preferred composition includes monoclonal anti-PrP 15B3 (12% w/w), T-BSA (58% w/w), PEG (12% w/w), carboxy PEG (8% w/w) and mouse brain homogenate (10% w/w added as solution).

The brain homogenate included for surface engineering is taken from transgenic 0/0 mice, which do not produce PrP, neither cellular nor infectious forms. For each analyte the composition of the mixture used for surface engineering needs optimisation with respect to amount and type of constituents.

Example 4 - Detection and purification of a PrP receptor

The biosensor system offers the invaluable advantage to visualize interactions of an immobilized protein (which would be PrP) with a ligand under nondenaturing conditions. This is exemplified by the interaction of PrP with the mAB 6H4 (see above Figure 3). As described in the introduction, several intracellular ligands have been characterized, however, their role in the normal function of PrP or in disease is not clear. From the cell surface location of PrP as well as other indirect evidence it is supposed that a PrP receptor should exist. Recombinant mouse PrP immobilized on a biosensor chip was incubated with membrane fractions from mouse brain. Membranes were prepared on a sucrose gradient as described (Oesch, 1994). In those experiments a 120 kDa PrP ligand was detected in membrane fractions. In order to analyze the specificity of interaction, it was blocked by the addition of monoclonal antibodies. Both mAB 6H4 and 34C9 recognize the region proposed to be involved in an interaction between PrP molecules. Membranes from PRP^{0/0} mice served as control, as it was known that the interaction of a membrane fraction with immobilized PrP was indeed due to PrP^{Sc} (also found in membranes).

Claims

1. An optical sensor unit for the specific detection, identification or accumulation of chemicals or biomolecules in an analyte, based on an integrated optical light pointer consisting of a laser light source, an optical detection module, and an integrated optical transducer chip whereon at least a layer of sensing biomolecules is bonded and whereby biospecific molecular interactions are identified by registration of changes in the effective refractive index.
2. An optical sensor unit according to claim 1, which comprises further a fluid handling system and a computer.
3. An optical sensor unit according to claim 1, in which the integrated optical transducer chip is of the smart planar optical transducer type.
4. An optical sensor unit according to claim 1, in which the transducer chip is of disposable nature.

5. An optical sensor unit according to claim 1, in which the optical transducer chip is made of a glass or organic polymer substrate (e.g. polycarbonate), is coated with at least one hard dielectric layer to form a waveguide and is covered with a layer of sensing biomolecules. 5
6. An optical sensor unit according to claim 5, in which the hard dielectric film is selected among silicon nitride, titanium dioxide, mixtures of silicon dioxide and titanium dioxide, ZrO_2 , HfO_2 , Nb_2O_5 and Ta_2O_5 . 10
7. An optical sensor unit according to claim 5, in which the layer of sensing biomolecules is covalently immobilized on the surface of the waveguiding layer by a method chosen among chemical surface grafting and photochemical immobilization. 15
8. An optical unit according to claim 5, in which the layer of sensing biomolecules is immobilized on the surface of the waveguiding layer by Langmuir-Blodgett techniques. 20
9. An optical unit according to claim 5, in which the layer of sensing biomolecules is passively absorbed on the waveguiding layer. 25
10. An optical sensor unit according to claims 7-9, in which the layer of sensing biomolecules is immobilized in a patterned fashion on individual areas by mask-assisted, mask-free patterning microstamping or microprinting procedures resulting in biosensor arrays. 30
11. An optical sensor unit according to claim 10, in which the immobilisation of the sensing biomolecules is further achieved by photoimmobilisation. 35
12. An optical sensor unit according to claim 6, in which the layer of sensing biomolecules is immobilized on individual areas by laser addressed covalent binding. 40
13. An optical sensor unit according to claim 12, in which the sensing biomolecules immobilized on individual sensing areas are different from each other. 45
14. An optical sensor unit according to claims 1-13 in which the sensing areas on the integrated optical transducer chip are arranged in vertical and horizontal rows. 50
15. An optical sensor unit according to claims 1 to 12, in which the integrated optical transducer chip is a disc and the sensing areas are aligned in one or several concentric circles or in a spiral arrangement. 55
16. An optical sensor unit according to claims 1 to 15, in which the immobilized sensing biomolecules are monoclonal or polyclonal antibodies, proteins or peptides thereof, agglutinins, lectins, polycarbohydrates, receptor proteins, low molecular weight haptens, nucleic acids.
17. An optical sensor unit according to claims 1 to 16, in which the sensing biomolecules are immobilized individually or as mixtures on distinct sensor areas to establish gradient patterns.
18. An optical sensor unit according to claims 1 to 17, in which the sensing biomolecules are antibodies which recognize prions.
19. An optical sensor unit according to claims 1 to 18, in which the integrated optical transducer surface contains recombinant, normal or disease-specific prion proteins.
20. An optical sensor unit according to claims 1 to 19, in which the surface of the integrated optical transducer is grafted with lipids, forming lipid bilayer surfaces, serving as membrane mimetic system for the integration of membrane receptor proteins.
21. A procedure for the use of an optical sensor unit according to claims 1 to 20, in which the signal is enhanced by enriching the analyte on the chip in a first step by binding to immobilized sensing biomolecules according to claims 16 to 20, and in a second step detected by binding to an other sensing biomolecule.
22. A procedure according to claims 1 to 21, in which the analyte is detected by complexing to an additional biomolecule in solution prior to the binding to the integrated optical chip.
23. A procedure according to claims 1 to 22, where the proper functioning of the integrated optical transducer is verified in situ.
24. A procedure according to claims 1 to 23, where the verification is performed before and after the measurement by means of varying the angle of incidence.
25. A procedure according to claims 1 to 24, where the verification is performed before and after the measurement by means of varying the wavelength of the light.
26. Applications utilizing the procedures according to claims 21 to 25 to identify the analytes which interact with immobilized sensing biomolecules after incubation with samples consisting of constituted flu-

ids. body fluids, homogenates or extracts of tissue specimen.

38. Applications according to claims 29-37, where the effector system is a fluid handling system.

27. Applications utilizing the procedures according to claims 21 to 25 to identify novel ligands to the biomolecules immobilized on the chip. 5

28. Applications utilizing the procedures according to claims 21 to 25 to simplify the detection of immunological responses or antigens specific for infectious diseases such as BSE, HIV, CJD, Hepatitis B, tropical diseases, toxic substances and drugs. 10

29. Applications using the procedures according to claims 21 to 25, where as a result of a specific reaction on the chip, the signal from the optical transducer is detected by a sensor resulting in a computable signal that is guided to a processing unit and subsequently to an effector system able to regulate, to start or to terminate one or more chemical or biochemical reactions. 15 20

30. Applications according to claim 29, where the regulated reaction is the same that causes the change of the output signal derived from the integrated optical transducer, resulting in a feedback to the reaction. 25

31. Applications according to claims 29-30, where the regulated reaction is repeated several or many times, resulting in cycles of reactions. 30

32. Applications according to claims 29-31, where the regulated reaction is in another sensor unit. 35

33. Applications according to claims 29-32, where several reactions are regulated between each other such that they follow a reaction chain, a reaction circle or other dependent reactions. 40

34. Applications according to claims 29-33, where the effector signal is a digital computable signal.

35. Applications according to claims 29-34, where one or more computable sensor signals are electronically processed by a computer such that the biosensor system is used as an interface between biological and electronical information. 45

36. Applications according to claims 29-35, where in a central processing unit input of biosensor systems is computed with other sensor systems of other modalities. 50

37. Applications according to claims 29-36, where after computing with particular software specific output results able to regulate specific reactions on biosensor systems. 55

Figure 1

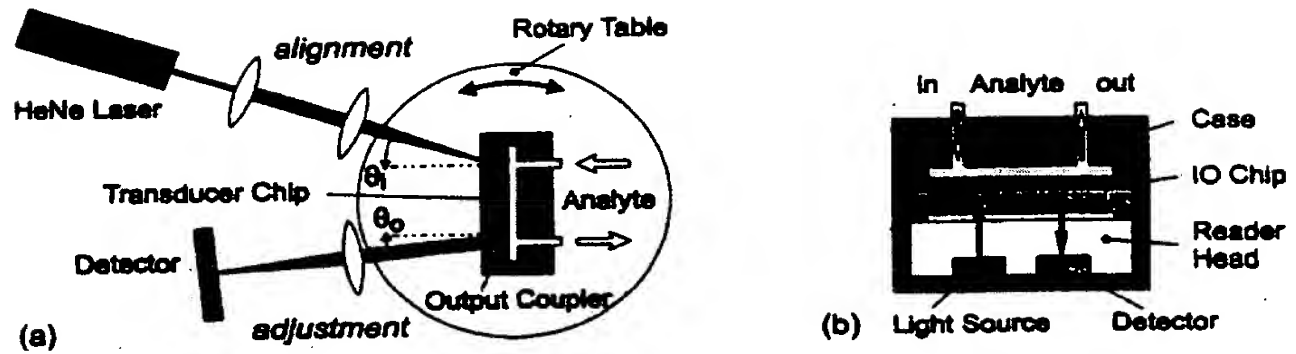
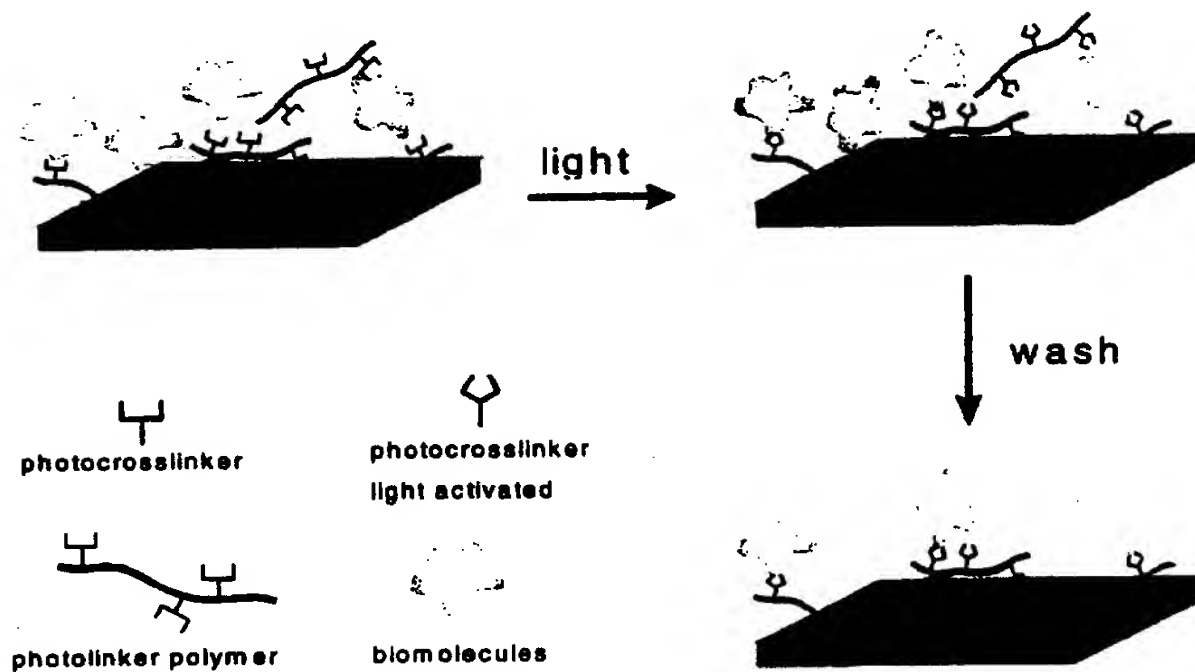


Figure 2.a



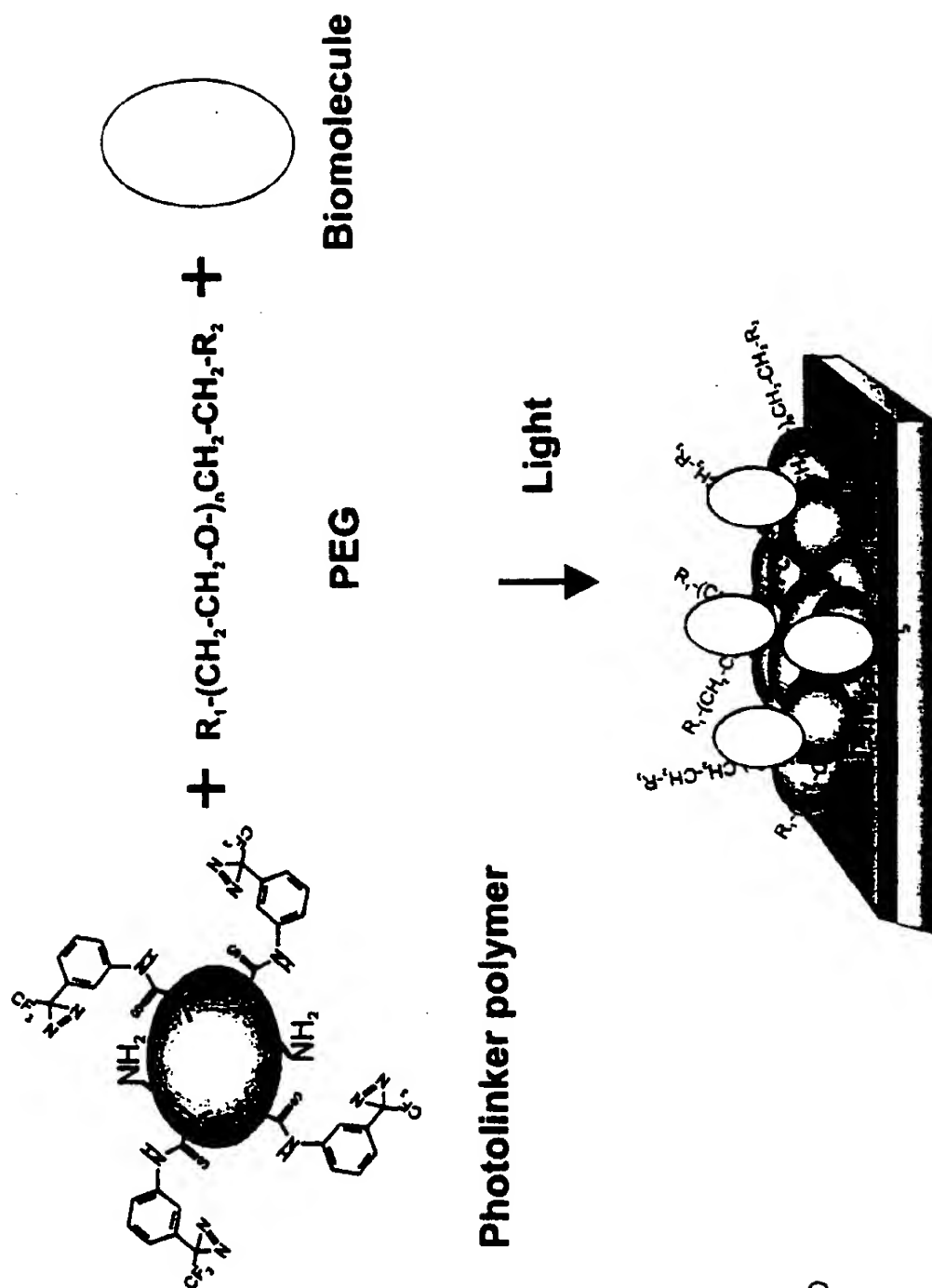


Figure 2.b

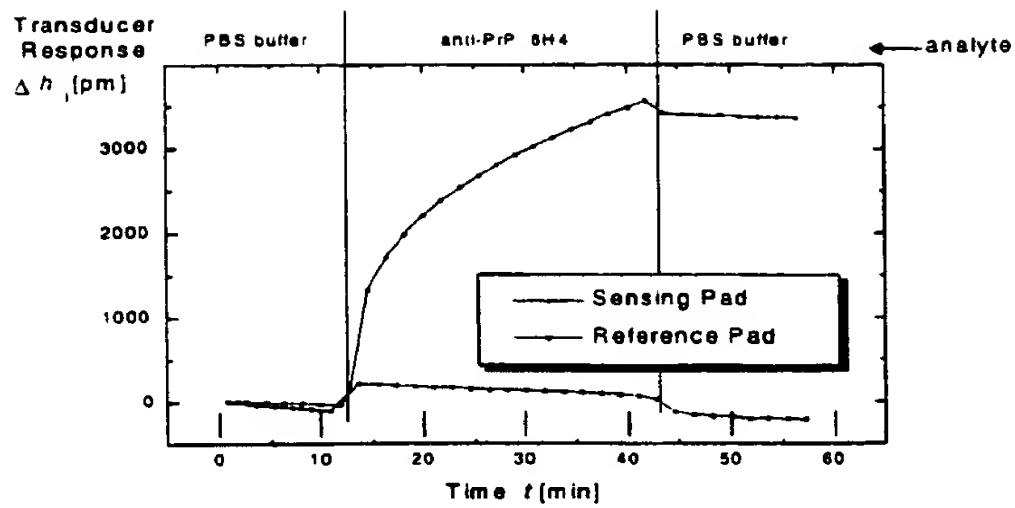


Figure 3

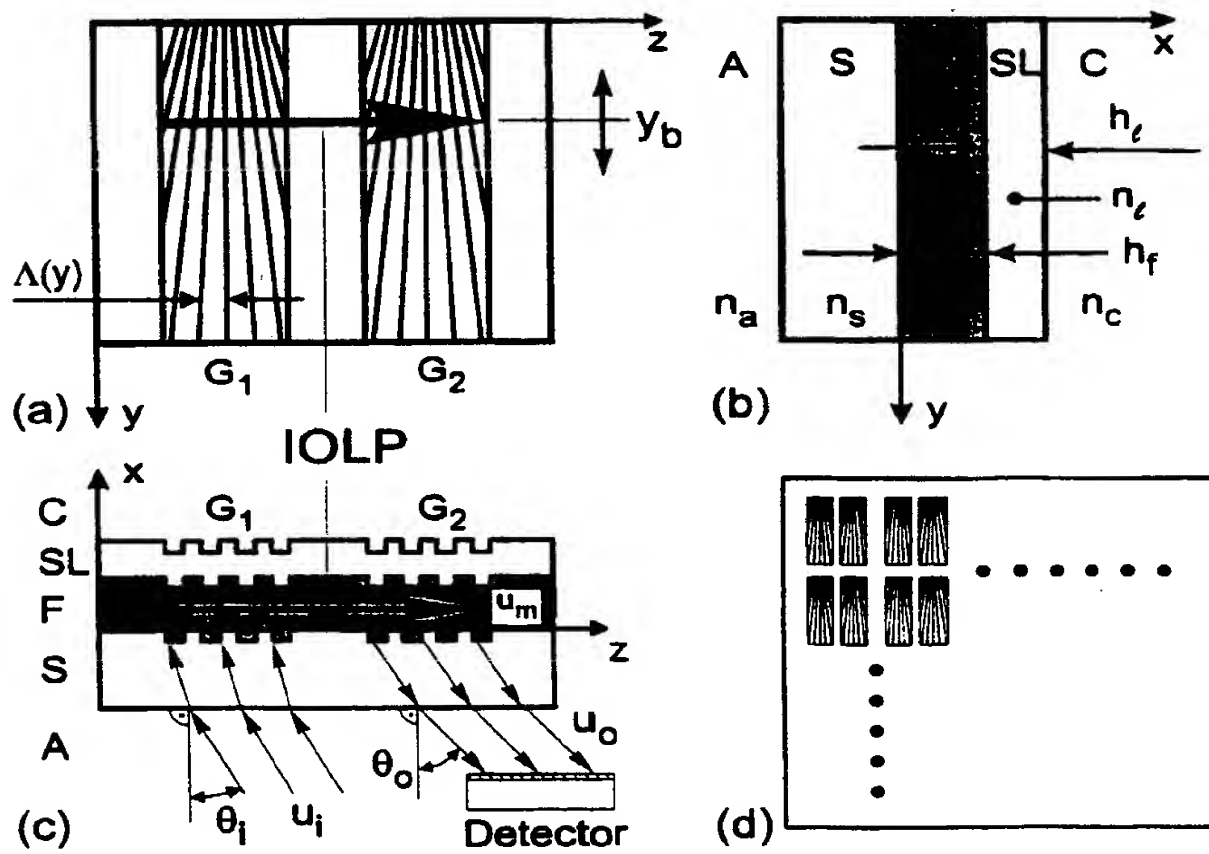


Figure 4

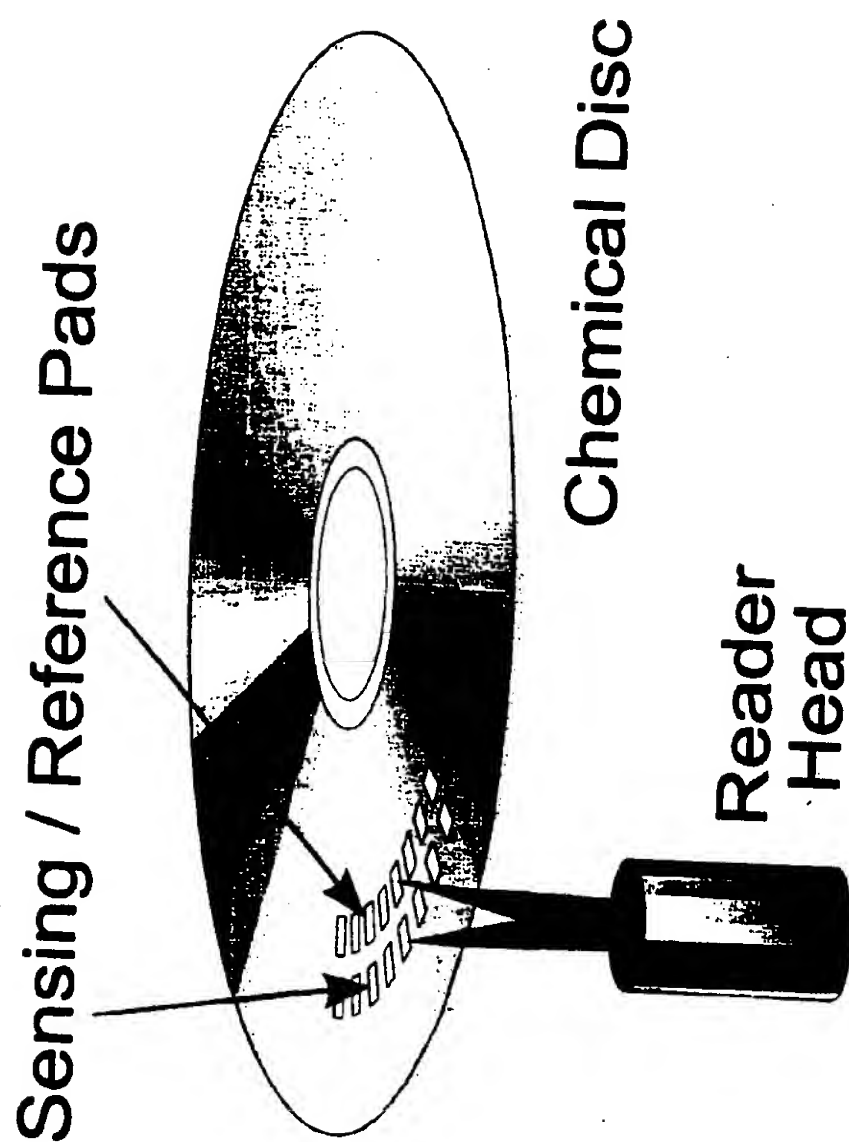


Figure 4. e

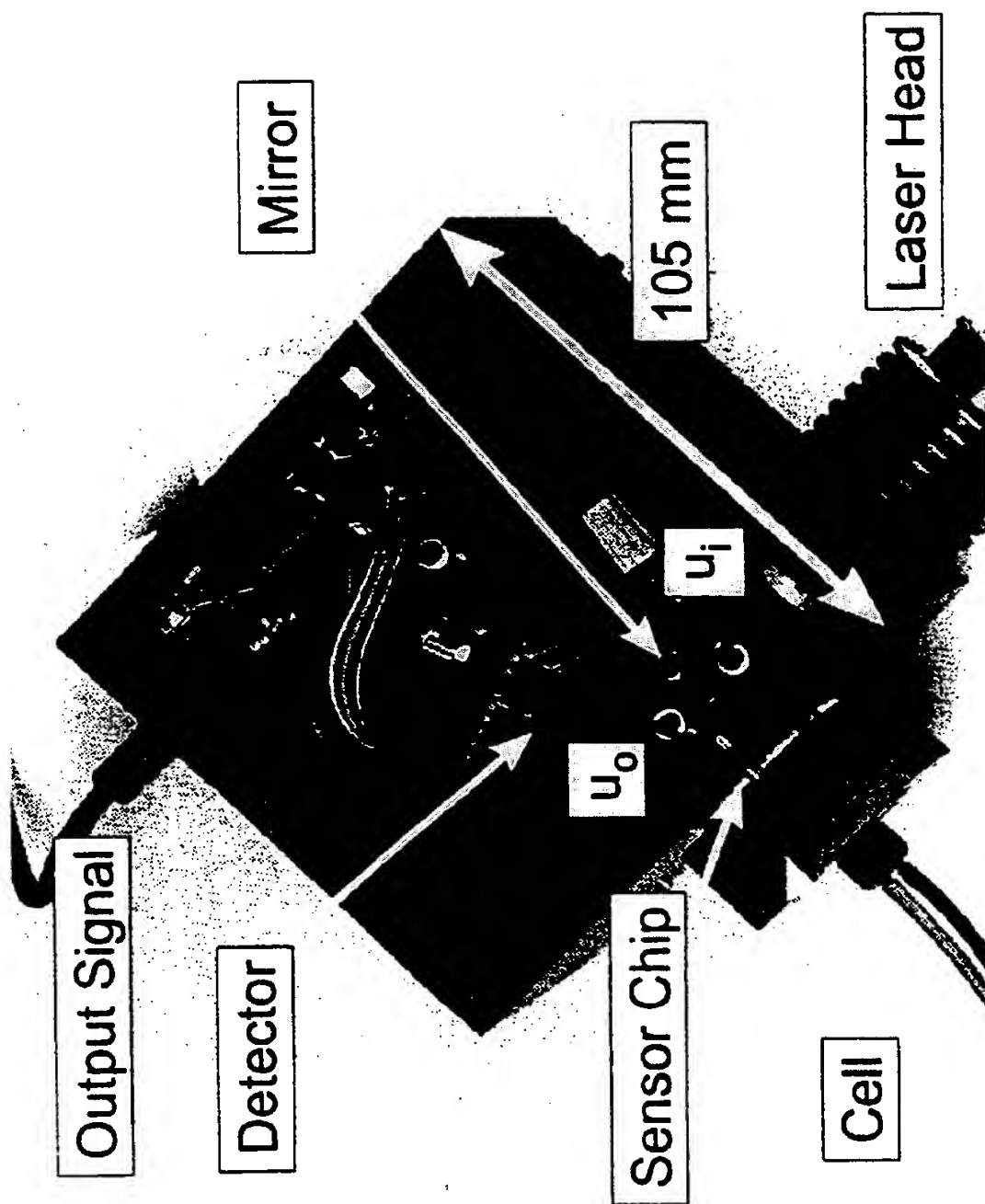


Figure 5



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 98 81 0508

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	M.T. GALE: "Polymer and III-V transducer platforms for integrated optical sensors" OPTICAL ENGINEERING, vol. 34, no. 8, August 1995, pages 2396-2406, XP000518235 BELLINGHAM US * page 2396, left-hand column, paragraph 1 * * page 2396, right-hand column, line 13 - page 2397, left-hand column, line 7 * * page 2397, right-hand column, line 22 - line 37 * * page 2400, right-hand column, paragraph 2 * * page 2402, left-hand column, line 1 - line 12 * * page 2402, left-hand column, line 28 - line 41 * * page 2404, left-hand column, line 11 - line 20 * * page 2404, right-hand column, last paragraph - page 2405, left-hand column, line 8; figures 1, 3A, 8, 11 *	1-6.16	G01N33/543
Y	---	7, 10, 11, 13-15, 20, 29, 34-36, 38	TECHNICAL FIELDS SEARCHED (Int.Cl.6) G01N
Y	WO 96 35940 A (CIBA-GEIGY) 14 November 1996 * page 5, line 8 - line 10 * * page 6, last paragraph - page 7, line 24 * * page 8, last paragraph - page 9, line 6 * * page 13, line 5 - page 14, last line * * page 15, line 10 - line 17 * * page 16, line 1 - line 8 * * page 18, line 3 - page 20, line 3 * * figures 2-5 *	7, 10, 11, 13-15, 20	
A	---	1-3, 5, 6, 16	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 7 October 1998	Examiner Thomas, R.M.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPF FORM 1503 03 82 (P04-001)



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 98 81 0508

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	US 4 204 037 A (DILL) 20 May 1980 * column 2, line 63 - column 3, line 17 * * column 9, line 25 - line 41 * * column 11, line 11 - line 27: figure 1 * ---	29 34-36, 38	
X	WO 97 12225 A (GEORGIA TECH) 3 April 1997 * page 2, line 4 - line 14 * * page 16, line 14 - line 17 * * page 17, line 3 - line 9 * * page 17, line 13 - line 19 * * page 21, last line - page 22, line 23 * * page 25, line 4 - line 5 * * figures 3C, 3D, 8B * ---	1, 2, 16, 23	
A	WO 89 10551 A (IGEN) 2 November 1989 * page 1, paragraph 1 * * page 8, line 19 - line 21 * ---	18, 19, 28	
A	DATABASE WPI Section Ch. Week 9046 Derwent Publications Ltd., London, GB: Class B04, AN 90-344994 XP002046986 & JP 02 249480 A (NGK SPARK PLUG) . 24 March 1989 * abstract * -----	29	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
The present search report has been drawn up for all claims			

EPC FORM 1503 (3.82) (P-C01)

Place of search THE HAGUE	Date of completion of the search 7 October 1998	Examiner Thomas, R.M.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document</p> <p>T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons S: member of the same patent family, corresponding document</p>		

11

○

○

